Early Journal Content on JSTOR, Free to Anyone in the World

This article is one of nearly 500,000 scholarly works digitized and made freely available to everyone in the world by JSTOR.

Known as the Early Journal Content, this set of works include research articles, news, letters, and other writings published in more than 200 of the oldest leading academic journals. The works date from the mid-seventeenth to the early twentieth centuries.

We encourage people to read and share the Early Journal Content openly and to tell others that this resource exists. People may post this content online or redistribute in any way for non-commercial purposes.

Read more about Early Journal Content at http://about.jstor.org/participate-jstor/individuals/early-journal-content.

JSTOR is a digital library of academic journals, books, and primary source objects. JSTOR helps people discover, use, and build upon a wide range of content through a powerful research and teaching platform, and preserves this content for future generations. JSTOR is part of ITHAKA, a not-for-profit organization that also includes Ithaka S+R and Portico. For more information about JSTOR, please contact support@jstor.org.

# A STUDY OF THE VIRUS OF RABIES, FREED FROM THE CELLS OF THE HOST AND FROM CONTAMINATING ORGANISMS.*

D. W. Poor and Edna Steinhardt.†

*(From the Research Laboratory of the New York Board of Health, New York City.)*

## CONTENTS.

I. Introduction.
II. Experimental Work.
  A. Methods of obtaining the virus from the submaxillary glands.
    1. Glycerin extraction.
    2. Aspiration.
  B. Virulence tests of glycerin virus.
  C. Virulence tests of submaxillary glands whose glycerin extracts are negative.
  D. Virulence tests of glycerin extracts of other organs.
  Comparison of the effect of glycerin on bacterial cultures of diphtheria and tubercle bacilli.
  E. Comparison of brain and gland virus.
    1. Viability.
    2. Effect of glycerin.
    3. Effect of rapid drying of brain and gland virus.
    4. Effect of combined drying and heat on brain and gland virus.
    5. Effect of heat.
    6. Effect of hydrochloric acid, bichloride of mercury and magnesium dioxid.
    7. Effect of dialysis.
    8. Effect of concentration.
    9. Filtration.
      *a*) Berkefeld N.
      *b*) Berkefeld V.
      *c*) Pasteur Chamberland F.
      *d*) Pasteur Chamberland B.
      *e*) Collodion sac.
  F. Attempts at cultivation.
  G. Microscopical examination.
  H. Attempts at agglutination.
III. Summary and Conclusions.

## I. INTRODUCTION.

Experimental work on rabies has largely been carried on with the virus as it occurs in the brain and spinal cord, where it is closely

---

* Received for publication June 9, 1913.

† This paper was presented before the Congress of Hygiene, Washington, D.C., September, 1912.

associated with the elements of the nerve tissue. Comparatively little has been done with the virus as it occurs in the salivary glands, where it is presumably in a more or less free state.

By the application of two new methods[1] we have been able to obtain a virus of rabies from the submaxillary glands in a comparatively pure state, freed from cells of the glands and unmixed with other organisms. Our work with this virus has been carried on with two objects in view: (1) the determination, if possible, of its nature, and (2) a comparison of this gland virus with the brain and cord virus.

If this comparison brought out marked physical differences, it seemed to us that this would suggest that the organism undergoes a cycle and exists in a different condition in the submaxillary gland from that in which it is found in the nervous system.

Of course, we have also used our pure virus for microscopical and cultural studies, but altho considerable work has been done in this direction, the results as yet have been inconclusive, and we are still continuing the experiments.

## II. EXPERIMENTAL WORK.

### METHODS OF OBTAINING THE VIRUS FROM THE SUBMAXILLARY GLANDS.

### GLYCERIN EXTRACT VIRUS.

The submaxillary glands from dogs, dead of street rabies, were used in both methods. Several years ago, one of us[2] found that the virus of rabies could be extracted with glycerin from the submaxillary glands of rabid dogs.

The glands were removed from the dog's head under aseptic precautions. After removal of the fibrous sheath, they were immersed in sterile neutral glycerin and allowed to stand in the icebox for six to twelve days. This glycerin extract retained its virulence when kept in the icebox for a very long time, in one experiment 191 days. No further work was done on the extract due to the limitations of experimenting with a glycerin virus.

In our present experiments, we found that altho virus may be extracted from the intact glands, more was obtained when the glands were cut in eight to ten pieces, and this was always done in the following work. The clear supernatant glycerin slightly colored by hemoglobin was pipetted off. This was usually found to be uncontaminated, and microscopical examination showed no formed cellular elements.

By our present method, dialysis through collodion sacs, we are able to get rid of glycerin and have our virus uncontaminated in any fluid we select. All the operations are carried on under aseptic conditions. The sac is sterilized, the glycerin extract contains no contaminating organisms, and as the collodion membrane does not permit the usual bacteria to pass through, the fluid in which the sac is placed for dialysis need not be sterile.

---

[1] Poor and Steinhardt, *Jour. Infect. Dis.*, 1913, 12, p. 202.

[2] Poor, *Proc. New York Path. Soc.*, 1906, 6, p. 85.

The dialysis of the glycerin takes place very rapidly. In running physiological salt solution, Ringer's solution, or distilled water, one and one-half hours are usually sufficient for the removal of the glycerin from four or five cubic centimeters of the virus. After dialysis, the virus is diluted, the dilution varying with the amount of water the glycerin has previously extracted from the tissue.

If a concentration is desired, the collodion sac may be used as a filter, as according to our experiments this virus does not pass through it, and, therefore, as much of the fluid as is desired may be removed. It is possible that a certain amount of the virus adheres to the side of the sac, so the concentration cannot be computed exactly. As shown by one of us,[1] proteins and probably salts in solution gradually pass through the sac after absorption reaches a certain degree, but the protein and salt contents of the fluid within the sac will be somewhat concentrated as the water passes through most rapidly. This fact must be borne in mind in interpreting some of the results of concentration.

The technic used for making collodion sacs was that described by Novy[2] and modified by Gorsline.[3] The sacs were made over a tube, which had a small opening in the bottom. This tube was turned several times in dilute collodion, and this collodion, when partially dried, was stripped off by forcing water through the tube. A glass tube was then inserted into the neck of the sac, and the junction strengthened by a ligature or coating of collodion.

The sacs were filled with distilled water, the tubes plugged with cotton, autoclaved at 105° C. for 15 minutes, allowed to cool, then emptied, immersed in sterile water and subjected to pressure (about three inches of mercury). If there was no evidence of leakage the sacs were used. The sacs were without flaws or air bubbles. For dialysis they were thick; for filtration thin. The virus obtained in this manner will be referred to as the *glycerin virus*.

### THE ASPIRATION VIRUS.

The glands after being cut in eight to ten pieces were placed in distilled water and subjected to a vacuum of 29 inches of mercury for an hour. The pieces of glands were then pressed in an ordinary meat press and the expressed fluid centrifuged. After centrifugation the supernatant fluid was pipetted off and added to the water in which the glands were aspirated. The aspiration fluid alone was found to contain the virus, but more was present on the addition of the pressed-out juice. By this method a suspension of the virus was obtained in distilled water which was stronger than the glycerin virus, but which was not sterile and which contained fragments of gland cells, blood cells, etc. Subsequent experiments showed that a Berkefeld filter held back bacteria and formed tissue elements, but the virus passed through. Thus by filtration we were able to get a sterile virus. This virus will be referred to as the *aspiration virus*.

---

[1] Steinhardt, *Jour. Infect. Dis.*, 1910, 7, p. 675.

[2] *Laboratory Work in Bacteriology*, Ann Arbor, 1899, p. 499.

[3] *Contrib. Med. Res.*, Ann Arbor, Mich., 1903, p. 390.

TABLE I.

VIRULENCE TESTS OF ASPIRATION GLAND VIRUS (STREET RABIES).

| Number of Test | Guinea-Pigs Inoculated | Number Infected | Individual Incubation | Average Incubation |
|---|---|---|---|---|
| | | | Days | Days |
| 1 | 3 | 3 | 8<br>9<br>8 | 8 1-3 |
| 2 | 2 | 2 | 8<br>10 | 9 |
| 3 | 2 | 2 | 9<br>9 | 9 |
| 4 (Two sets of glands aspirated together) | 3 | 3 | 9<br>8<br>8 | 8 1-3 |
| 5 (Four sets of glands aspirated together) | 2 | 2 | 8<br>8 | 8 |
| 6 (Two sets of glands aspirated together) | 2 | 2 | 10<br>10 | 10 |
| 7 (Three sets of glands aspirated together) | 3 | 3 | 16<br>19<br>25 | 20 |

## VIRULENCE TESTS OF THE GLYCERIN EXTRACT VIRUS.

Glycerin extracts of the submaxillary glands from 45 rabid dogs (street virus) were tested for virulence. The brains of most of these dogs showed Negri bodies. As Table 2 shows, 33 of the extracts were virulent or 73.33 per cent; the inoculated guinea-pigs developed rabies with incubation periods ranging from 8 to 82 days. Of the non-virulent extracts the longest period in which the animals were kept was 69 days. This is unfortunate as we now realize that had we kept the animals longer, more cases of rabies might have developed.

In a few cases where the smears of the fresh brain showed no positive Negri bodies, the gland extract, either glycerin or aspiration virus, proved virulent with a short incubation period, shorter than the brain inoculation. Therefore, occasionally this virus may be used for diagnostic methods, especially in those cases in which the brain is not fresh, and with the aspiration virus of the glands the Berkefeld filtrate can be used.

The glycerin extract was diluted with twice the amount of salt solution and about one-third of a cubic centimeter was inoculated subdurally into each guinea-pig. Altho the guinea-pigs showed clinical rabies, the brain smears were always examined for Negri

TABLE 2.

VIRULENCE TESTS OF GLYCERIN EXTRACTS OF SUBMAXILLARY GLANDS OF RABID DOGS (STREET VIRUS).

VIRULENT EXTRACTS.

| Number of Test | Number of Days in Glycerin | Number of Guinea-Pigs Inoculated | Number Infected | Individual Incubation Period (Onset of Symptoms) | Average Incubation Period |
|---|---|---|---|---|---|
| | | | | Days | Days |
| 1 | 14 | 3 | 3 | 8<br>13<br>14 | 11 2-3 |
| 2 | 16 | 2 | 2 | 11<br>11 | 11 |
| 3 | 10 | 3 | 2<br>(1 died 24 hrs. after inoculation; not rabies) | 10<br>11 | 10 1-2 |
| 4 | 4 | 3 | 3 | 14<br>10<br>10 | 11 1-3 |
| 5 | 10 | 3 | 3 | 16<br>18<br>18 | 17 1-3 |
| 6 | 4 | 3 | 3 | 10<br>10<br>10 | 10 |
| 7 | 10 | 3 | 3 | 10<br>12<br>14 | 12 |
| 8 | 13 | 3 | 2<br>(1 died in 4 days; not rabies) | 13<br>13 | 13 |
| 9 | 6 | 3 | 2<br>(1 died in 7 days; not rabies) | 55<br>65 | 60 |
| 10 | 6 | 3 | 2<br>( dead in 6 days; not rabies) | 13<br>13 | 13 |
| 11 | 8 | 3 | 3 | 7<br>16<br>17 | 13 1-3 |
| 12 | 8 | 3 | 3 | 9<br>11<br>11 | 10 1-3 |
| 13 | 5 | 3 | 3 | 13<br>13<br>22 | 16 |
| 14 | 6 | 3 | 2<br>(1 discharged) | 15<br>21 | 18 |
| 15 | 6 | 3 | 3 | 21<br>21<br>21 | 21 |
| 16 | 7 | 3 | 1<br>(1 dead in 1 day; 1 dead in 6 days; not rabies) | 82 | 82 |
| 17 | 7 | 4 | 1<br>(2 dead in 4 days; not rabies 1 discharged after 82 days) | 34 | 34 |
| 18 | 7 | 3 | 2<br>(1 dead in 4 days; not rabies) | 12<br>20 | 16 |
| 19 | 7 | 3 | 1<br>(2 dead in 4 days; not rabies) | 12 | 12 |

TABLE 2—*Continued.*

| Number of Test | Number of Days in Glycerin | Number of Guinea-Pigs Inoculated | Number Infected | Individual Incubation Period (Onset of Symptoms) | Average Incubation Period |
|---|---|---|---|---|---|
| 20 | 7 | 3 | 3 | 18<br>18<br>19 | 18 1-3 |
| 21 | 7 | 3 | 2<br>(1 discharged) | 13<br>13 | 13 |
| 22 | 7 | 3 | 1<br>(1 dead in 4 days; 1 dead in 5 days; not rabies) | 15 | 15 |
| 23 | 13 | 2 | 1<br>(1 dead in 5 days; not rabies) | 14 | 14 |
| 24 | 6 | 3 | 1<br>(1 dead in 2 days; 1 dead in 4 days; not rabies) | 16 | 16 |
| 25 | 8 | 3 | 2<br>(1 dead in 24 days; not rabies) | 10<br>12 | 16 |
| 26 | 6 | 2 | 1<br>(1 discharged in 180 days) | 21 | 21 |
| 27 | 7 | 3 | 2<br>(1 discharged) | 10<br>10 | 10 |
| 28 | 7 | 3 | 1<br>(1 dead in 25 days; not rabies. 1 discharged) | 11 | 11 |
| 29 | 8 | 3 | 2<br>(1 discharged) | 17<br>18 | 17 1-2 |
| 30 | 12 | 3 | 1<br>(2 discharged after 138 days) | 14 | 14 |
| 31 | 14 | 3 | 2<br>(1 discharged) | 9<br>10 | 9 1-2 |
| 32 | 12 | 3 | 1<br>(1 missing after 4 days. 1 discharged 36 days) | 18 | 18 |
| 33 | 14 | 3 | 1<br>(2 missing after 6 days) | 18 | 18 |

bodies and if not found reinoculations were made into fresh guinea-pigs. The possibility of a contaminating infection giving paralytic symptoms closely resembling rabies was brought to our notice in some of our animals and a report of that work will be published later.

That a single glycerin extraction may not remove all the virus from the glands was shown by the following experiment. In the virulent extracts, the glycerin extract of Virus 15 was withdrawn from the glands after six days and fresh glycerin added. This second glycerin extract was removed after eight days. The first extract gave rabies to guinea-pigs (3 in each set) with an average incubation period of 21 days, while the second gave rabies with an

NON-VIRULENT EXTRACTS.

| Number of Test | Number of Days in Glycerin | Number of Guinea-Pigs Inoculated | Number Infected | Date Discharged Days |
|---|---|---|---|---|
| 1 | 50 | 2 | None | 37 |
| 2 | 23 | 2 | None<br>(1 dead in 18 days. No Negri bodies found. Reinoculation negative) | 37 |
| 3 | 14 | 3 | None | 60 |
| 4 | 4 | 3 | " | 53 |
| 5 | 14 | 3 | " | 47 |
| 6 | 7 | 3 | " | 47 |
| 7 | 13 | 3 | " | 47 |
| 8 | 14 | 2 | " | 69 |
| 9 | 8 | 3 | " | 2 discharged in 85 days |
| 10 | 6 | 3 | None<br>(1 died in 64 days; not rabies) | 2 discharged in 85 days |
| 11 | 10 | 3 | None<br>(1 dead in 26 days; not rabies) | 2 discharged in 138 days |
| 12 | 14 | 3 | None | 172 |

average of twenty-four and two-thirds days. This showed but a slight dilution of the virus.

## VIRULENCE TESTS OF SUBMAXILLARY GLANDS WHOSE GLYCERIN EXTRACTS ARE NEGATIVE.

That the action of glycerin on the glands is mainly that of sucking out the virus from the tubules is seen from the following experiment. The glands from two rabid dogs had given a non-virulent glycerin extract. They were removed from the glycerin and ground up in a mortar with salt solution, a difficult operation owing to the tough and slippery condition of the glands. These emulsions, extracted in the icebox overnight, proved to be virulent, giving rabies with an average incubation period of 29 days in one case, and of 12 days in the other. In another set of glands in which the glycerin extract had an incubation period of 56 days, the ground-up glands gave rabies in 10 days.

On the other hand, in some glands much virus was present in the ducts, as we have shown by a second extraction of virus with fresh glycerin giving rabies with a short incubation, confirming the fact of the variability of the virulence of the saliva of rabid animals.

## VIRULENCE TESTS OF GLYCERIN EXTRACTS OF OTHER ORGANS.

*Rabid brains.*—Glycerin extracts were made of 11 fixed virus brains (rabbit) of which only four were virulent, and the glycerin extracts of four street virus brains (dog) were all non-virulent (see Table 3), showing in all only four virulent extracts from 15 brains, that is 26.66 per cent virulence compared with 73.33 per cent of the glycerin gland extract.

When one considers that the brain, at least for fixed virus, is the culture medium for the rabies organism, which is present in much greater numbers in this situation than in the glands, one would expect a greater number of virulent brain extracts, unless, as is undoubtedly the case, the virus is here intimately associated with the nerve cells. Indeed the virulent extracts from the brain virus might be accounted for by minute particles of the brain substance being torn off by the action of the glycerin (the brain being a more delicate tissue than the gland) or by the extraction of the virus from the blood and lymphatic vessels and spaces, and as we have shown, the virus, even in the submaxillary glands, is not always in an extractable state.

TABLE 3.

VIRULENCE TESTS OF GLYCERIN EXTRACTS, RABID BRAINS OF RABBIT FIXED VIRUS AND DOG STREET VIRUS.

| Number of Test | Material Extracted | Number of Days in Glycerin | Filtered | Guinea-Pigs Inoculated | Number Infected | Average Incubation Period | Discharged |
|---|---|---|---|---|---|---|---|
| | | Days | | | | | Days |
| 1...... | Fixed virus rabbit brain | 9 | Not filtered | 2 | None | None | 36 |
| 2...... | " " " " | 12 | Not filtered | 3 | " | " | 53 |
| 3...... | " " " " | 12 | Through cotton | 3 | " | " | 53 |
| 4...... | " " " " | 9 | a Not filtered | 3 | 2 | 11½days | 1 in 46 |
| | | | b Through 1 layer of filter paper | 3 | 1 | 13 " | 2 in 41 |
| 5...... | " " " " | 9 | a Not filtered | 3 | 2 | 9 " | 1 in 46 |
| | | | b Through 1 layer of filter paper | 3 | 2 | 10½ " | 1 in 41 |
| 6...... | " " " " | 11 | Through 1 layer of hardened filter paper | 3 | 2 | 8 " | 1 in 46 |
| 7...... | " " " " | 11 | Through 1 layer of hardened filter paper | 3 | None | ........ | 46 |
| 8...... | " " " " | 10 | Through 1 layer of hardened filter paper | 3 | 1 | 8 " | " |
| 9...... | " " " " | 10 | Through 1 layer of filter paper | 3 | None | ........ | " |
| 10...... | " " " " | 10 | Through 1 layer of filter paper | 3 | " | ........ | " |
| 11...... | " " " " | 10 | Through 1 layer of filter paper | 3 | " | ........ | " |
| 12...... | Street virus (dog) brain | 16 | Through cotton | 3 | " | ........ | 33 |
| 13...... | " " " " | 16 | Through cotton | 3 | " | ........ | " |
| 14...... | " " " " | 16 | Through 1 layer of filter paper | 3 | " | ........ | " |
| 15...... | " " " " | 12 | Through 1 layer of filter paper | 3 | " | ........ | " |

In one case where the brain of a rabid dog (street virus) showed Negri bodies, part of the Ammon's horn, suprarenal capsule, and sciatic nerve were immersed in glycerin; the extracts were tested for rabies and all were non-virulent. In three cases of street rabies the glycerin extract of the pancreas was non-virulent.

These experiments are too few from which to draw conclusions; they were carried out to try the glycerin extraction on other organs which have been found virulent in rabies. Whether the aspiration method would have shown the virus to be in an extractable condition in these organs is to be decided by further experiments.

### COMPARISON OF THE EFFECT OF GLYCERIN ON DIPHTHERIA AND TUBERCLE BACILLI.

Emphasis is usually laid on the resistance of the parasite of rabies to the action of glycerin, and this resistance is frequently considered to be opposed to the virus being of a bacterial nature.

We have made comparative tests with cultures of tubercle and diphtheria bacilli. Ten cubic centimeters of pure sterile glycerin were put on the cultures (agar and egg media) so that the growth was completely immersed. These were placed in the icebox for two weeks in the dark.

The supernatant glycerin from the diphtheria culture was dialyzed and planted in broth; incubation at 37.5° C. gave a vigorous growth of diphtheria organisms. The examination of the stained slides showed the bacilli in glycerin to be perfectly stained altho somewhat small. After dialysis, the stained slides from the non-incubated fluid showed bacilli indistinguishable from those from a fresh culture.

On cultures of bovine tubercle bacilli on egg media about 10 c.c. of glycerin was added so that the growth was more than covered and placed in the icebox in the dark. Three weeks later the supernatant glycerin was clear; there were no visible particles in it. Three cubic centimeters of this glycerin were dialyzed in running 0.8 per cent salt solution in a collodion sac for two hours. This dialyzed fluid was inoculated into guinea-pigs and produced general tuberculosis in two to three weeks.

After an interval of 13 months in the icebox, a portion of the growth of the same cultures, still covered by glycerin, was removed emulsified and injected into guinea-pigs. These died of general tuberculosis, showing that the tubercle bacilli had not lost their virulence even after being acted upon by the glycerin for 13 months. The staining showed no diminution in the acidfastness of the organisms, and transfers gave growth on egg medium.

These two cultures, diphtheria and tubercle bacilli, are organisms which are not killed by drying, and as the action of glycerin is largely the withdrawal of water from that with which it is in contact, our results are not surprising. We find that these bacteria are not destroyed by glycerin, the tubercle bacillus having a resistance as great as the virus of rabies. This resistance, therefore, cannot be considered as necessarily indicating a protozoal virus.

We are now starting experiments on the action of glycerin in the cold and dark on other organisms, that may possibly have some bearing on the virus of rabies.

### COMPARISON OF BRAIN AND GLAND VIRUS.

There are various hypotheses as to the nature of the virus of rabies. Some investigators consider it of bacterial origin with possible spore formation, while others favor a protozoal theory with a definite cycle. There are no Negri bodies in the gland virus tho they exist in the brain. To test the question of a possible difference in the physical state of the virus in the two situations, brain and gland, differences which might throw light on the supposed cycle or spore formation, comparative experiments were made on viability, virulence, action of glycerin, the effect of rapid drying, of heat, of combined drying and heat, of dialysis, and of some chemical agents. In no instance was there any definite difference in the reaction of the two viruses to any of the tests, showing that as far as our work goes at present, it all indicates a similar physical condition of the virus of rabies in the gland and in the brain.

*Viability.*—Gland virus, aspirated in distilled water, was kept in an icebox at 7–8° C. for 15 days, at the end of which time it had become offensive through bacterial contamination. It was then filtered through a Berkefeld V candle, a filtrate being obtained which produced rabies in guinea-pigs with an incubation of 14 days.

This filtrate kept at room temperature and exposed to light for 45 hours had lost its virulence completely.

An emulsion of fixed virus in distilled water exposed to the same conditions for the same length of time was markedly weakened, the incubation of the injected animals being increased to 10 days, five days longer than the controls. Continuing the exposure to light and room temperature for 96 hours destroyed the virulence of the brain emulsion.

This experiment gives but a rough comparison as there were undoubtedly many more organisms present in the fixed virus emulsion than in the gland virus filtrate, and, as is known, the number of organisms present, and the amount of protein and salts in the emulsion greatly modify the results.

In another experiment the Berkefeld filtrate from an aspiration gland virus kept in the icebox for nine days had lost its virulence while the unfiltered virus kept in the same manner for the same length of time and then filtered proved virulent. This is in accord with the experiments of Di Vestea[1] on brain virus. He found that the filtrate of fixed virus died out in the icebox within a week, while the original unfiltered virus retained its strength for a long time.

Di Vestea also found the filtrate to be more easily destroyed by heat and glycerin, and from these results he concludes that the filtrate does not contain the entire rabic virus, that the virus is of a protozoal nature, of which only the young forms are filterable, and that these young forms are more easily destroyed by heat and glycerin and conservation. Di Vestea's facts are undoubtedly correct but there is another and simpler interpretation for them. As we have said before, in all comparative tests, variations in the number of organisms present and the concentration of proteins and salts of the material tested vary the results greatly. There are undoubtedly fewer organisms in a filtrate than in the original unfiltered virus and as shown by one[2] of us unless one filters a sufficient amount to saturate or coat the filter, the filtrate will contain considerably less proteins than the original solution. The fact that Di Vestea found his filtrate less resistant than his original emulsion and the confirmation of that fact in this work with gland virus is more probably explained by the fewer number of organisms and the lesser amount of proteins in the filtrate than by a protozoal cycle.

---

[1] *Ann d'ig. sper.*, 1905, 15, p. 147; *La Med. Ital.*, 1904, 2, p. 241.

[2] Steinhardt, *Jour. Med. Research*, 1904, 13, p. 409.

*Comparative virulence.*—The incubation period of the gland virus is usually much shorter than that of the brain in street rabies. Subdural inoculations in guinea-pigs with emulsion of dog's brain, street virus, generally produce rabies with an incubation period of 14 days, as compared with the usual eight to nine days with the gland virus. This difference in incubation period could be explained in several ways: (1) the lesser virulence of organisms in the brain virus; (2) the lesser number of organisms in the brain virus; (3) the presence of brain substances in the inoculations with this virus: this brain substance might act either directly or by causing a slowness of absorption.

One experiment with the glycerin extract of a dog's brain, street virus, showed an incubation period of only nine days. As in our other glycerin extract tests a piece of brain of a rabid dog, street virus, was put in glycerin for six days and then some of the supernatant glycerin was removed. This glycerin was not filtered and contained any small particles of brain matter, so we could not consider it as an extract and omitted it from Table 3. Three guinea-pigs were inoculated with it using the same methods and dilutions as for the other extracts. Of these, one contracted rabies with an incubation of nine days, the other two were negative and discharged 108 days later. Thus at least for this one case the street brain was as virulent as the gland extract. As yet we have not made a further study of this question and until more work has been done, no statement can be made as to the comparative strength of the street virus in the two situations.

The fixed virus is undoubtedly the stronger, judging from the shorter incubation period, even at great dilutions.

*Action of glycerin.*—The action of glycerin, according to Marie,[1] in destroying the virus of the rabid brain is a slow one if the mixture is kept at icebox temperature and is dependent on the amount of brain tissue, and it is difficult to fix the limits of this action.

---

[1] *L'étude expérimentale de la rage* (Dorin et Fils, editors), 1909, p. 124.

Poor found, as quoted before, that under similar conditions the gland extract retained its virulence a long time. In one case at the end of 191 days, guinea-pigs inoculated with the glycerin extract showed rabies after 12 days' incubation.

In the present experiments, we have found a gradual weakening of the glycerin gland virus, kept in the icebox. This weakness was shown by a prolonged incubation period or complete avirulence.

In one instance, the glycerin that had been on the glands for 10 days was inoculated into three guinea-pigs. It infected all with an average incubation period of 12 days. The same extract kept on the gland in the icebox, 37 days later infected two out of three guinea-pigs with an average incubation of 19 days.

In one case we tested a virulent glycerin extract virus after six months' preservation in the icebox and found it had lost its virulence.

When kept in the icebox, the action of glycerin on the virus of rabies in the brain or in the gland, altho a variable one, brought out no difference between the virus from these two sources.

*Rapid drying.*—It is an established fact that brain virus if dried rapidly retains its virulence. That gland virus under similar conditions is equally as resistant is shown by the following comparative experiment.

As Harris and Schackell[1] state that it is the concentration of salts in the drying which causes the destruction of the rabies virus, to partially eliminate this factor, a preliminary dialysis in distilled water was done with the brain virus. An emulsion of fixed brain virus was made with distilled water put in a collodion sac and dialyzed in running distilled water for two hours. Part of this dialyzed fluid was kept in the icebox as control. The remainder was spread in a thin layer and dried in a vacuum of 29 inches of mercury over sulfuric acid. After 16 hours the drying was complete and the material was rubbed up in salt solution to its original volume. Subdural inoculations in guinea-pigs with the same doses of this and the control emulsion produced rabies in five to six days in both cases.

The glands from two rabid dogs (street virus) were aspirated separately. These two aspiration gland viruses were put under the same conditions as the brain virus.

---

[1] *Jour. Infect. Dis.*, 1911, 8, p. 47.

The results here also showed no destruction of the virus, as inoculations with both the dried and the control gland virus gave rabies to guinea-pigs in nine and one-half days.

TABLE 4.

EFFECT OF RAPID DRYING ON EMULSIONS OF FIXED BRAIN VIRUS AND ASPIRATION GLAND VIRUS (STREET).

| Number of Test | Material Inoculated | Guinea-Pigs Inoculated | Individual Incubation | Average Incubation |
|---|---|---|---|---|
| | | | Days | Days |
| 1 (Control fixed virus) | Fixed virus brain emulsified in distilled water and dialyzed | 3 | 5<br>5<br>7 | 5 2-3 |
| 2 (No. 1 dried) | 5 c.c. of No. 1 dried in vacuum, then emulsified to original volume with salt solution | 3 | 5<br>5<br>7 | 5 2-3 |
| 3 (Control fixed virus) | Fixed virus brain emulsified in salt solution | 2 | 5<br>5 | 5 |
| 4 (No. 3 dried) | 5 c.c. of No. 3 dried in vacuum, then emulsified to original volume with distilled water | 2 | 1 dead in 7 | 7 |
| 5 (Control gland virus A) | Aspiration gland virus (street) A | 2 | 8<br>10 | 9 |
| 6 (No. 5 dried) | Same as gland virus A. Dried in vacuum for 10–15 hours, then emulsified with salt solution | 3 | 9<br>13<br>10 | 10 2-3 |
| 7 (Control gland virus B) | Aspiration gland virus (street) B | 3 | 9<br>9<br>10 | 9 1-3 |
| 8 (No. 7 dried) | Same as gland virus B. Dried in vacuum for 10–13 hours, then emulsified with salt solution | 3 | 10<br>8<br>9 | 9 |

*Effect of combined rapid drying and heat.*—An emulsion of fixed virus and a glycerin gland virus was dialyzed in distilled water for an hour. Very thin layers of each were evaporated to dryness while exposed to 37° C. for six hours. In each case the virus was killed while the controls were fully virulent. No difference in the nature of the two could be detected by this means.

*Effect of heat.*—In regard to the thermal death point of the virus in rabid brain emulsions, Marie[1] states that the length of time required to weaken and finally destroy the virus by heat varies according to the thickness of the emulsion, or to express it otherwise, according to the number of specific germs present.

---

[1] *Loc. cit.*

It was not possible to standardize the emulsions for comparison, but our experiments showed no marked difference between the fixed brain virus and gland street virus, altho the original gland virus was the weaker as shown by the longer incubation when inoculated into guinea-pigs.

Exposure for 10–15 minutes to 48–50° C. weakened both viruses, while the same temperature for 30 minutes killed the gland virus, and greatly weakened the fixed brain virus, only one guinea-pig out of three inoculated developing rabies and that with a prolonged incubation.

Both viruses were destroyed when kept at 60° C. for 30 minutes as shown by Table 5.

TABLE 5.

EFFECT OF HEAT.

| Number of Test | Material Inoculated | Heated | Guinea-Pigs Inoculated | Individual Incubation | Average Incubation |
|---|---|---|---|---|---|
| 1........ | Glycerin extract gland virus dialyzed in distilled water for one hour (Control) | .............. | 3 | 12 days<br>11 "<br>11 " | Days<br>11 1-3 |
| 2........ | Fixed virus brain emulsion dialyzed same as above | .............. | 3 | 5 "<br>"<br>1 dead in 1 day; not rabies | 4 |
| 3........ | Fixed virus brain emulsion | 48–50° C. for 15 min. | 3 | 5 days<br>6 "<br>6 " | 5 2-3 |
| 4........ | Glycerin extract gland virus dialyzed for 1½ hours in salt solution | 48–50° C. for 10 min. | 2 | 1 dead in 60 days<br>1 dead in 10 days; not rabies | 60 |
| 5........ | Fixed virus brain emulsion | 48–50° C. for 30 min. | 3 | 9 days; 2 discharged in 62 days | 9 |
| 6........ | Glycerin extract gland virus dialyzed for 1½ hours in salt solution | 48–50° C. for 30 min. | 3 | Discharged in 62 days | .......... |
| 7........ | Glycerin extract gland virus dialyzed for 1½ hours in salt solution | 60° C. for 10 min. | 3 | Discharged in 48 days | .......... |
| 8........ | Fixed virus brain emulsion dialyzed same as above | 60° C. for 30 min. | 3 | Discharged in 48 days | |
| 9........ | Glycerin extract gland virus dialyzed for 1½ hours in salt solution | 60° C. for 30 min. | 3 | Discharged in 48 days | .......... |

*Effect of chemicals.*—The chemicals tested on the brain, centesimal emulsion of fixed virus, and the aspiration gland virus were bichlorid of mercury, hydrochloric acid, and magnesium dioxid.

With none of these was there any marked difference in the resistance of the two viruses. A 1–1000 solution of bichlorid of mercury killed both viruses. A 1–1000 solution of hydrochloric acid killed the brain virus and markedly weakened the gland virus, only one guinea-pig out of three contracting rabies with a prolonged incubation. Magnesium dioxid was tried because of its oxidizing effect. In the strength of one-fourth of a gram to four cubic centimeters of the virus, it produced but little effect.

*Effect of dialysis.*—Novy and Knapp[1] have shown that dialysis kills fixed rabies virus apparently through the extraction of certain salts and that the time required depends on certain factors such as the thickness of the emulsion, rate of the flow of water, etc. Our results were similar to theirs, tho not identical, probably owing largely to the fact of our using much stronger emulsions. We found, e.g., that the dialysis of two cubic centimeters of either brain or gland virus in 200–300 c.c. of standing water had but little effect after 18 hours, but that dialysis of the gland virus in running water for 18 hours killed it. Brain virus on the other hand, while it was markedly weakened, was not completely killed. Dialysis for 43 hours killed the fixed virus emulsion used by us. There were many more tissue elements as well as organisms in the brain virus emulsion used than in the glycerin gland virus which undoubtedly accounts for the longer time required for killing the former virus.

*Effect of concentration.*—Some of the filtrate from a Berkefeld V candle was evaporated in a vacuum over $H_2SO_4$ to one-fifth its bulk—the operation requiring four hours. The control in a narrow tube sealed with paraffin was placed in the apparatus so that conditions of light and temperature would be the same. Three guinea-pigs were inoculated from the concentrated virus as well as from the control and also the concentrated virus diluted to its original volume. The result was that while the pigs inoculated with the control died with an average incubation of 20 days, those from the concentrated virus developed with an incubation of 32 days, while only one pig from the concentrated virus subsequently diluted, developed after 20 days.

This suggests a harmful concentration of salts or proteins, such as is believed by Harris[1] to cause the reduction of virulence in the Pasteur cords.

---

[1] *Jour. Infect. Dis.*, 1906, 3, p. 291.

TABLE 6.

EFFECT OF DILUTION AND CONCENTRATION.

| Number of Test | Material Inoculated | Number of Guinea-Pigs Inoculated | Number Infected | Individual Incubation | Average Incubation |
|---|---|---|---|---|---|
| | | | | Days | Days |
| 1*a*....... | Control virus aspiration gland No. 1 | 3 | 3 | 9<br>9<br>8 | 8 2-3 |
| 1*b*....... | Above virus diluted 1 to 4 | 3 | 3 | 8<br>9<br>11 | 9 1-3 |
| 2*a*....... | Aspiration gland virus No. 2 | 2 | 2 | 8<br>8 | 8 |
| 2*b*....... | Above virus diluted 1 to 8 | 4 | 4 | 12<br>9<br>9<br>9 | 9 3-4 |
| 3*a*....... | Aspiration gland virus No. 3 | 2 | 2 | 10<br>10 | 10 |
| 3*b*....... | Aspiration gland virus No. 3 diluted 1 to 10 | 4 | 3 | 9<br>11<br>17 | 12 1-2 |
| 3*c*....... | Aspiration gland virus No. 3 diluted 1 to 20 | 4 | 4 | 9<br>11<br>13<br>15 | 12 |
| 4*a*....... | Aspiration gland virus No. 5 | 2 | 1 | 8<br>(1 dead in 2 days; not rabies) | 8 |
| 4*b*....... | Aspiration gland virus No. 5 diluted 1 to 30 | 3 | 3 | 10<br>9<br>12 | 10 1-3 |
| 5*a*....... | Aspiration gland virus No. 6 | 3 | 3 | 16<br>19<br>25 | 20 |
| 5*b*....... | Aspiration gland virus No. 6 concentrated over sulfuric acid in vacuum (5.5 c.c. to 1.2 c.c.) | 3 | 3 | 19<br>33<br>45 | 32 1-3 |
| 5*c*....... | Same virus as 5*b*. After concentration diluted to original volume with salt solution | 3 | 1 | 20 | 20 |

*Effect of filtration.*—In 1903 Remlinger[2] first showed that fixed virus was filterable. He emulsified the entire brain of a fixed virus rabbit in 400 c.c. of water and filtered the emulsion through a Berkefeld V filter under pressure. Ten rabbits were inoculated with from one-half to one cubic centimeter each of the filtrate.

---

[1] *Jour. Infect. Dis.*, 1912, 10, p. 369.

[2] *Ann. de l'Inst. Pasteur*, 1903, 17, p. 834.

Three of these rabbits or 30 per cent developed rabies. The following year Marie,[1] using three different Berkefeld candles, was unable to pass the virus through in any instance. In the same year, 1904, however, Bertarelli and Volpino[2] successfully filtered fixed virus through a Berkefeld V filter five times out of seven trials. They failed to pass it through a Chamberland F. Celli and du Blasi[3] had similar results. In 1904 also Di Vestea[4] made extensive experiments on filtration. He used fixed virus and prepared the emulsions with great care, emulsifying the brains with the aid of quartz sand. He filtered with from two to three atmospheres pressure and inoculated his animals with large amounts of the filtrate, i.e., one-half to one cubic centimeter each. His results were as follows:

| | Experiments | Virulent Filtrates | Per Cent |
|---|---|---|---|
| Berkefeld V | 19 | 14 | 75 |
| Berkefeld N | 1 | 1 | ................ |
| Chamberland F | 8 | 2 | 25 |
| Chamberland B (Filtrate from Chamberland F) | 1 | 0 | ................ |
| Maasen Filter (Filtrate from Berkefeld) | 1 | 1 | ................ |

The literature contains very little regarding the filtration of gland virus. In one instance a virulent filtrate was obtained by Bertarelli and Volpino, who passed it through three layers of paper. It is also recorded that the virus in human saliva was passed in one instance through a Berkefeld candle.

Our experiments were made with Berkefeld V and N, Chamberland F and B, and with a collodion filter. The aspiration virus from the glands of from two to four dogs were used in each experiment. The results were as follows:

| | Experiments | Virulent Filtrates | Per Cent |
|---|---|---|---|
| Berkefeld V | 4 | 4 | 100 |
| Berkefeld N | 1 | 1 | ................ |
| Chamberland F | 4 | 3 | 75 |
| Chamberland B | 2 | 0 | ................ |
| Collodion Sac | 2 | 0 | ................ |

---

[1] *Loc. cit.*

[2] *Centralbl. f. Bakteriol., I Orig.*, 1904, 37, p. 51.

[3] *Ann. d'ig. sper.*, 1904, 3, p. 603.

[4] *Riv. d'ig.*, 1905, 16, p. 158; *La Med. Ital.*, 1904, 2, p. 241.

In the collodion sac and the first Chamberland B experiment, the virus unfortunately proved to be quite weak. In the second Chamberland B experiment, however, a strong virus was obtained, but inoculations with the filtrate failed to show symptoms of rabies after a lapse of six weeks.

In order to get some idea of the amount of virus held up by the filter, a series of dilutions of the fluid to be filtered was made as follows: dilutions of 1 to 4, 1 to 8, 1 to 10, 1 to 20, and 1 to 30. It was found that the average incubation of the guinea-pigs inoculated was increased in a surprisingly regular manner, according to the degree of the dilution of the virus. For example, the dilution of 1 to 4 gave an incubation of nine and one-third days, as against eight and two-thirds days for the undiluted virus, an increase of two-thirds of a day only. A dilution of 1 to 30 gave an incubation of ten and one-third days as compared with eight days for the original virus, an increase of two and one-third days (see Table 6). The average duration of incubation, then, may be taken as a rough index of the dilution of virus. All of the guinea-pigs inoculated with the Berkefeld filtrates developed rabies. That more of the virus passes through after saturation of the filter is seen from the following experiment. A small Berkefeld V candle was used. The first five cubic centimeters which passed constituted the first filtrate, the next 12 c.c., the second filtrate. The four guinea-pigs inoculated with the first filtrate developed rabies with an average incubation period of 21 days. Those with the unfiltered material diluted eight times developed in nine and one-half days and the undiluted control in eight days. Comparing the incubation of the pigs injected with the second filtrate, twelve and one-half days, with that of the pigs injected with the control diluted 1 to 8, nine and one-half days, it is fair to assume that in this case the virus was diluted by filtration more than eight times. Again in the finer Berkefeld, Grade N, the average incubation of the pigs injected with the second filtrate was fifteen and one-half days, and that produced by the virus diluted 1 to 20 was only eleven and one-half days. In a further attempt to determine the dilution caused by filtration, a Berkefeld V candle was shaved to about one-half its thickness. In this case a weak virus diluted 1 to 20 gave an incubation of 17

TABLE 7.

FILTRATION THROUGH BERKEFELD V FILTER.

| Number of Test | Material Inoculated | Number of Guinea-Pigs | Number Infected | Individual Incubation | Average Incubation |
|---|---|---|---|---|---|
| | | | | Days | Days |
| 1........ | Glycerin extract gland virus (Control) dialyzed | 5 | 2 | 12<br>18 | 15 |
| 2........ | First 5 c.c. of filtrate of Test 1 | 5 | 0 | ................ | Discharged 3 months later, not rabid |
| 3........ | Last (30 to 35 c.c.) of the filtrate of Test 1 | 5 | 1 | 56 | 56 |
| 4*a*....... | Aspiration gland virus (4 sets of glands) control diluted 1 to 8 | 4 | 4 | 12<br>9<br>9<br>8 | 9½ |
| 4*b*....... | Virus of Test 4 undiluted | 2 | 2 | 8<br>8 | 8 |
| 5........ | Virus of Test 4 passed through filter (first 5 c.c. of filtrate) | 4 | 4 | 18<br>26<br>14<br>26 | 21 |
| 6........ | Last 12 c.c. of the filtrate of Test 4 | 5 | 4 | 12<br>14<br>13<br>11 | 12½ |
| 7........ | Aspiration gland virus (3 sets of glands) passed through the filter | 3 | 3 | 16<br>19<br>25 | 20 |
| 8........ | Virus of Test 7 kept in icebox 6 days, showed evidence of bacterial contamination, passed through filter | 3 | 2 | 1 dead in 3 days; not rabies<br>14<br>15 | 14½ |

*Tests 1, 2, and 3.*—The glycerin extract of two sets of rabid glands was dialyzed in one and one-half hours and then passed through a new Berkefeld V filter with the aid of a vacuum pump. The first five cubic centimeters, called the first filtrate, were inoculated in guinea-pigs; the next 25 c.c were discarded (used to coat or saturate the filter) and the last five cubic centimeters were called the last filtrate and inoculated into guinea-pigs. The original virus was very weak, only two out of five guinea-pigs contracting rabies in Control Experiment 1. The last filtrate was virulent but not the first.

*Tests 4a, 4b, 5, and 6.*—Aspiration gland virus of four sets of rabid glands, amounting to 50 c.c. (much hemoglobin and very cloudy), was passed through a new Berkefeld V filter with the aid of a vacuum pump. The first five c.c., called the first filtrate, were inoculated into guinea-pigs; the next 12 c.c., called the last filtrate, were also inoculated. The filter was then blocked. The last filtrate proved more virulent than the first.

*Tests 7 and 8.*—Aspiration gland virus of three sets of rabid glands which were kept in the icebox four to six days was passed through a new Berkefeld V filter[1] by the aid of a vacuum pump. The first 15 c.c. were used to saturate the filter and the following seven cubic centimeters used for the experiment in Test 7. In Test 8 part of the original unfiltered virus used in Test 7 which had remained in the icebox for six days and showed evidence of bacterial contamination was passed through a new Berkefeld V filter with the aid of a vacuum pump. Eight cubic centimeters were used to saturate filter and the following six cubic centimeters, clear but of a reddish color and offensive odor, were injected into guinea-pigs, and proved virulent.

---

[1] New filters, generally the small size, were used in all filtrate experiments. The filter was washed in distilled water, then boiled in 0.4 per cent salt solution for 20 minutes. Before filtration was started the filter was partially drained by the aid of the vacuum. All filters were tested with a suspension of bacteria, usually pyocyaneus or diphtheria and proven perfect.

days while the filtrate from the shaved filter gave 16 days, practically the same, so that it may be assumed that the shaved filter diluted the virus about 20 times. The filtrates from the Chamberland filters gave correspondingly increased incubation. Thus in one test, two out of four pigs inoculated developed rabies with an average incubation of 33 days, as against eight and one-third days for the control. In a second test two out of five pigs developed with an average incubation of 47 days as against 11 days for the control. The filters were tested with a suspension of pyocyaneus bacilli or other bacteria which was mixed with the virus to be filtered. In no case did the bacilli pass through.

Comparing our results with the filtration of gland virus, with those obtained by others in the filtration of the fixed brain virus, one may infer that the greater regularity with which the gland virus passes (that is, 100 per cent in the Berkefeld as against 75 per cent, and 75 per cent in the Chamberland F as against 25 per cent obtained by Di Vestea) is due to the freer condition of the virus in the glands. That the organism is not necessarily smaller in this location is seen from the fact that the fixed virus did pass the Chamberland filter, and even in one instance, according to Di Vestea, the Maasen filter.

TABLE 8.

FILTRATION THROUGH BERKEFELD N FILTER.

| Number of Test | Material Inoculated | Number of Guinea-Pigs | Number Infected | Individual Incubation Days | Average Incubation Days |
|---|---|---|---|---|---|
| 1........ | Aspiration gland virus (2 sets of glands); control diluted 1 to 20 | 4 | 4 | 13<br>14<br>10<br>9 | 11½ |
| 2........ | Virus of Test 1 filtered through Berkefeld N | 4 | 4 | 14<br>16<br>16<br>16 | 15½ |

Aspiration gland virus of two sets of rabid glands was passed through a new Berkefeld N filter with the aid of a vacuum pump. The first 15 c.c. were used to saturate filter. The next 10 c.c. were tested in Test 2 and proved virulent.

A culture of *B. alvei* was used for the test of the filter, and the filter proved bacteria proof.

TABLE 9.

FILTRATION THROUGH PASTEUR CHAMBERLAND F.

| Number of Test | Material Inoculated | Number of Guinea-Pigs | Number Infected | Individual Incubation | Average Incubation |
|---|---|---|---|---|---|
| | | | | Days | Days |
| 1...... | Aspiration gland virus (3 sets glands). Control | 5 | 4 | 8<br>10<br>9<br>12<br>1 dead in 2 days, not rabies | 9 3-4 |
| 2...... | Virus of Test 1 passed through filter | 5 | 1 | 26 | 26<br>(4 pigs discharged 4 months later, not rabid) |
| 3...... | Aspiration gland virus (4 sets glands). Control | 2 | 1 | 11<br>1 pig disappeared | 11 |
| 4...... | Virus of Test 3, first 12 c.c. filtrate, passed through small filter | 5 | 2 | 46<br>48 | 47 |
| 5...... | Virus of Test 3, first 20 c.c. of filtrate, passed through large filter | 6 | ........ | 1 dead in 10 days, not rabies<br>*1 dead in 17 days | Other pigs discharged 4 months later, not rabid |
| 6...... | Aspiration gland virus (2 sets glands). Control | 3 | 3 | 9<br>8<br>8 | 8 1-3 |
| 7...... | Virus of Test 6 passed through large filter and first filtrate used | 4 | 2 | 27<br>39 | 33 |
| 8...... | Virus of Test 6 passed through large filter and last filtrate used | 5 | ........ | 1 dead in 5 days<br>1 dead in 40 days<br>No Negri bodies seen | Other pigs discharged 4 months later, not rabid |

* Guinea-pigs (4) inoculated from this brain died in two days. The brain was then put into glycerin and three days later three more pigs were inoculated from this material. Two died on the next day and the other was discharged four months later, not rabid.

*Tests 1 and 2.*—Aspiration gland virus of three sets of rabid glands passed through a new Chamberland F filter. Fifty cubic centimeters of the extract were put through the filter of which 35 c.c. were recovered and used in Test 2. Pyocyaneus broth culture used for efficiency of filter, filtrate sterile.

*Tests 3, 4, and 5.*—Aspiration gland virus of four sets of rabid glands was divided and passed through two new Chamberland F filters, one small size and one large. Filtration with the aid of a vacuum pump. In Test 4, 12 c.c. were passed through small filter of which four cubic centimeters were recovered. In Test 5, 20 c.c. were passed through large filter and seven cubic centimeters recovered. Pyocyaneus broth culture used for efficiency of filter, filtrate sterile.

*Tests 6, 7, and 8.*—Aspiration gland virus of two sets of rabid glands was passed through a new Chamberland F filter, large size, with the aid of a vacuum pump. The filtration was slow. The first five cubic centimeters to pass through, called first filtrate, were used in Test 7 and proved virulent. The next 15 c.c. were used to saturate filter and discarded. The last five cubic centimeters, constituting the last filtrate, were tested for virulence in Test 8 and proved non-virulent. A broth culture of diphtheria was used for the efficiency of the filter. Filtrate sterile.

TABLE 10.

PASTEUR CHAMBERLAND B FILTRATION.

| Number of Test | Material Inoculated | Number of Guinea-Pigs | Number Infected | Individual Incubation | Average Incubation |
|---|---|---|---|---|---|
| 1...... | Aspiration gland virus (5 glands from 3 dogs). Control | 2 | 1 | 1 dead, not rabies.<br>7 days | 7 days |
| 2...... | Virus of Test 1 filtered through large Pasteur B filter | 5 | ........ | 1 dead, 3 days } Not<br>1 " 104 " } ra-<br>1 " 107 " } bies | Other pigs discharged 4 months later, not rabid |
| 3...... | Aspiration gland virus (Control) 1–20 dil. | 3 | 3 | 12 days<br>12 "<br>17 " | 13 2-3 days |
| 4...... | Virus of Test 3 filtered through large Pasteur B filter | 5 | ........ | ..................... | Discharged 4 months later, not rabid |
| 5...... | Virus of Test 1*a*, Table 11, filtered through small Pasteur B filter | 5 | ........ | ..................... | Discharged 4 months later, not rabid |

*Tests 1 and 2.*—Aspiration gland virus of five rabid glands from three dogs was passed through a new Pasteur B filter with the aid of a vacuum pump. Before filtration, 25 c.c. of the virus was mixed with one cubic centimeter of a broth suspension of pyocyaneus and proved filter bacteria proof. The filtrate proved non-virulent.

*Tests 3 and 4.*—Aspiration gland virus diluted one to twenty in salt solution to which two loopfuls of a moist agar culture or pyocyaneus had been added was passed through a new large Pasteur B filter with the aid of a vacuum pump. Seventy-five cubic centimeters were filtered through in 15 minutes, then 25 c.c. of undiluted virus were further filtered through. As the filter had not been completely drained in the beginning 115–120 c.c. were recovered, and, inoculated in the guinea-pigs, proved non-virulent.

*Test 5.*—Aspiration gland virus was filtered through a shaven Berkefeld V filter (used in Test 1*a*, Table 11). This filtrate was shown to be virulent. It was refiltered through a new Pasteur B filter, small size; about 12 c.c. were passed through of which six cubic centimeters were recovered and proved non-virulent.

TABLE 11.

FILTRATION THROUGH COLLODION SAC.

| Number of Test | Material Inoculated | Number of Guinea-Pigs | Number Infected | Individual Incubation | Average Incubation |
|---|---|---|---|---|---|
| 1*a*....... | Aspiration gland virus No. 1*a* filtered through shaven Berkefeld V filter. Control | 3 | 2 | Days<br>17<br>17 | 17 days |
| 1*b*....... | Aspiration gland virus No. 1*a* filtered through a collodion sac | 5 | 0 | ................ | Discharged 3 months later; not rabid |
| 1*c*....... | Aspiration gland virus No. 1, unfiltered, diluted 1 to 20. Control | 3 | 3 | 12<br>12<br>17 | 13 2-3 days |
| 1*d*....... | Same virus as No. 1*c* filtered through a collodion sac | 5 | 0 | ................ | Discharged 3 months later; not rabid |

*Tests 1a and 1b.*—Part of the aspiration gland virus that was filtered through a shaven Berkefeld V filter was used in Test 1*a*. Then 3.5 c.c .of it were filtered through a collodion sac with the aid of a vacuum of 2.5″ to 3.″ The filtration required two and one-half hours. The filtrate tested in 1*b* proved non-virulent.

*Test 1d.*—Dilution of virus 1 to 20 used in Test 1*c* filtered through under same conditions as 1*b*. Filtration for 3.5 c.c. took five and one-half hours. Filtrate non-virulent. The sacs were tested by pressure before and after filtration and no leakage found.

## ATTEMPTS AT CULTIVATION.

In the first method tried, the spinal cord of a fixed virus rabbit four days after subdural inoculation was used. At this time the virus has reached the cervical end but has not extended to the lumbar extremity. The cord was quickly removed from the canal by the Oshida method, and blocked into previously sterilized paraffin of 46° melting point, the whole block being then immediately solidified in ice water. In this way a partial anaerobic condition was obtained, a condition considered by Marie to be desirable. At the end of 10 days not only had the virus not extended to the lower end, but it had died out in the cervical end.

Attempts were made to grow the virus or at least to get an idea of its viability, by making use of the technic of Harrison's method of tissue growing *in vitro*. It was found that apparently the brain cells retained their vitality at incubator temperature for several days, in one instance as long as 21 days. This was inferred from the perfect staining of the Nissl bodies. However, in spite of the ganglion cells remaining alive, small portions of the fixed virus brains imbedded in plasma, agar, or in simple saline solution, lost their virulence after seven days at 37° C.

On the other hand, in the only instance in which street virus brain was tried, it was found that the virulence was retained after eight days at 37° C.

Portions of normal guinea-pig brain inoculated with weak emulsion of fixed virus showed no virulence after six days. Normal brain was also inoculated in a similar manner with gland virus, but here also there was no evidence of virus at the end of six days. Unfortunately in this experiment a glycerin extract virus was used which proved to be very weak. From our subsequent work, as well as from clinical observations, we would conclude that the most promising virus to use for this purpose would be the strong aspiration virus freed from bacteria by passage through a Berkefeld filter. This would avoid the objection to brain virus for the cultivation of the rabic organism made by Marie on the ground that at incubator temperature the brain tissue develops substances detrimental to the virus.

## MICROSCOPICAL EXAMINATION.

The glycerin extract dialyzed in distilled water or in salt solution, even after concentration through a collodion sac or by evaporation in vacuum, gave a product which, while containing the organism, showed remarkably little in the way of formed elements of any kind. The aspiration virus when filtered through a Berkefeld V candle showed many small granules, of which some were evidently from the secreting cells of the gland, as they were seen also in extracts from the normal gland. Many examinations were made of both of these viruses with the dark field (1–12 and 1–16 lenses and 4 and 8 ocular), also unstained in light field and stained by the various intensive and special methods. In all cases small granules easily seen in the unstained preparations and taking most of the stains with comparative ease were found; but these were found in the extracts of normal glands too, altho it occasionally seemed to us that there were more granules in the rabid extracts. Sometimes a ring was seen with a central point and, rarely, slender, slightly curved structures, suggesting cilia, were found. We were unable, however, to identify any of our findings as the specific organism.

## ATTEMPTS AT AGGLUTINATION.

Anti-rabic serum was produced by immunizing guinea-pigs and rabbits with fixed virus, according to Marie's method. This serum was tested for its agglutinative properties against the rabid gland extracts. Since rabid nerve tissue of the rabbit was used as the antigen, and the rabid gland extract of the dog for the agglutination test, it was hoped we might get a specific agglutination of the organism of rabies, with tissue reactions excluded, a result unobtainable by the use of rabid nerve tissue for both immunization and agglutination.

Preliminary experiments showed in the extracts of the glands of both normal and rabid dogs, an agglutination of granules, apparently greater in the rabid extracts than in the controls. The results were not sufficiently definite to warrant any conclusions. Further work is being carried on in this line and also with the opsonic technic and results will be given in a later paper.

## III. SUMMARY AND CONCLUSIONS.

By our two new methods, glycerin extraction and aspiration, we have been able to obtain the virus of rabies from the submaxillary glands, free from the cells of the host and from contaminating organisms. We were thus able to study the virus in a fairly pure state and to compare it with the virus as it exists in the nervous tissue.

Forty-five glycerin extracts of the submaxillary glands of dogs (street rabies) were tested for virulence by subdural inoculations in guinea-pigs. Thirty-three or 73.33 per cent were virulent, the average incubation periods ranging from eight to 82 days. A single extraction of glycerin did not always extract all the virus, for in some cases tested, a second glycerin extract of the same glands also proved virulent. An emulsion from glands which had given a non-virulent extract was virulent, showing the action of the glycerin in extracting the virus to be largely mechanical, a sucking out of the virus from the tubules and ducts.

Glycerin extracts of 11 fixed virus brains (rabbit) were virulent in only four instances, while four glycerin extracts of street virus brain (dog) were all non-virulent, showing in all four virulent extracts from 15 rabid brains, that is, 26.66 per cent virulence as compared with 73.33 per cent of the glycerin gland extract. Considering the great virulence of fixed virus brains, one would expect a greater number of virulent glycerin brain extracts. The result, however, need not necessarily argue a difference of form of the virus in this situation, but rather indicates that the virus is here more intimately associated with the nerve cells, while in the glands it is probably largely a drawing out from the ducts. The few virulent brain extracts might have been due to very minute particles of the brain substance, the brain being more friable than the gland, or to the extraction of the virus from the blood and lymphatic vessels and spaces.

A few glycerin extracts made from the suprarenal capsule, sciatic nerve, and pancreas from the dog (street virus) were all non-virulent.

The aspiration virus of the rabid submaxillary glands was always virulent, usually with an incubation period of from eight to nine days.

The resistance of the virus of rabies to glycerin so frequently taken as suggesting a protozoal origin has been found by us to be paralleled by diphtheria and tubercle bacilli. Cultures of these bacteria, which were subjected to glycerin in the cold and dark, diphtheria bacilli for two weeks, tubercle for 13 months, on dialysis and transplantation, gave vigorous growths. Their staining properties were not impaired, and the tubercle bacilli were fully virulent; the diphtheria culture was not tested for virulence. As the action of glycerin is largely that of the extraction of water or drying and both the cultures withstand drying tho neither has a spore stage, the results are not surprising.

We find, therefore, that these bacteria are not destroyed by glycerin, the tubercle bacillus having a resistance as great as the virus of rabies, which resistance cannot therefore be considered as necessarily indicating a protozoal virus.

In comparing the virus as it exists in the brain and in the gland, this fact must be borne in mind: with viruses which cannot be standardized slight differences in reactions to either physical or chemical agents are not to be interpreted as indicating a difference in their physical or biological state, as variations in the number of organisms present in the concentration of proteins and salts vary the results with the reactions of the different emulsions.

Comparative experiments on the brain and gland virus for viability, for the effect of rapid drying, heat, combined drying and heat, filtration, dialysis, action of glycerin, of magnesium dioxid, of bichlorid of mercury and of hydrochloric acid, have shown no sufficient difference to indicate either a protozoan cycle or a spore bacterial form in the virus of rabies in these two situations.

In our filtration experiments with the gland virus we obtained 100 per cent virulent filtrates with the Berkefeld filters and 75 per cent with the Pasteur Chamberland F filter. Di Vestea, working with brain emulsion, secured only 75 per cent positive filtrates from the Berkefeld filter and 25 per cent from the Pasteur Chamberland F. This difference in results need not argue a smaller form of the organism in the gland. It may be due to the virus being less intimately associated with the cells of the host in this situation, as was previously suggested by the fact of glycerin extract of rabid brains being virulent in only 26.66 per cent as compared with the 73.33 per cent of virulence of the glycerin gland extracts.

Whether this would indicate that the chromatin elements of the Negri body may be the true rabic parasite, the outer portion being a degeneration product of the nerve cell, we are at present unprepared to say. The following points are to be considered in this connection. First, the structure known as the complete Negri body could not pass through the filter; second, some of the minute, inner cell-structures might pass; third, the small central structures might easily exist free in the gland and escape identification. In this connection, it is interesting to note that working in conjunction with Dr. R. A. Lambert,[1] we have been able to produce inclusions in ganglion cells, closely resembling the outer portion of Negri bodies, by allowing normal nerve cells to slowly degenerate in plasma at 37° C. according to Harrison's technic for the growing of tissues *in vitro*.

Many factors also are to be considered in judging the results of filtration. The question of the coating or saturation of filter is sometimes of great importance, even for the passage of proteins in solution as shown by one of us in complement filtration, and as our present work has shown in the Berkefeld filtrate. Therefore, in any question of filtration the amount filtered, the first or last filtrate tested, the dilution of the organism and of the foreign protein and the amount of salts in the fluid to be filtered, the pressure used, the possible concentration of the filtrate by evaporation, centrifugation, precipitation, or possible agglutination, all are important factors toward the end results. So the comparison of filtration experiments unless done under identical conditions cannot be taken as absolute proof of difference in size.

The fact of a virus passing through even the Chamberland Pasteur F filter does not mean that it is invisible with our present powers of magnification. The organism of peripneumonia, the classic example of a filtrable virus, readily passes through this filter but is easily seen by the use of the dark field and oil immersion.

Our attempts at cultivating the virus of rabies even with the *in vitro* method have not as yet been successful.

From our microscopic examinations we conclude that the virus of rabies as it exists in the submaxillary gland, altho possibly visible, is probably of no distinctive form. By the use of various staining methods, by frequent examinations of many viruses with both light and dark fields, with high magnification, no distinctive formed elements were seen. In all cases small granules were readily found. Occasionally there were rings with a central point and cilia-like structures, but these were also found in the control extracts of normal submaxillary glands. Occasionally it seemed to us that there were more granules in the rabid than in the normal extracts, but no conclusions could be drawn. Experiments on the possibility of obtaining a specific agglutination of the virus of rabies as it occurs in the gland extract from an antirabic serum, obtained by the injection of rabid brain, were tried. By this method it was hoped to exclude tissue reactions. In some cases it seemed as if there were greater clumping of granules in the rabid extracts than in the control normal extracts. Further work is now being carried on along this line and with the opsonic technic with the possibility of a specific phagocytic action locating the virus in the leukocytes. Until these experiments are completed no definite conclusions can be drawn concerning the granules.

---

[1] *Jour. Infect. Dis.*, 1912, 11, p. 459.

In closing, we wish to emphasize the following points:

1. The advantage of using the gland secretions in the study of rabies because they contain the organism freed from tissue cells. This is an advantage for the microscopical study of the virus, for the study of its physical properties, and for its use in growing experiments.

2. The two easy methods for obtaining bacterially sterile virus from the glands.

3. The resistance of bacteria to glycerin which is as great for the tubercle bacillus as for the virus of rabies.

4. The regularity with which the gland virus passes the Berkefeld filter and the large percentage of virulent filtrates with the Chamberland Pasteur F filter.

5. The similarity in the nature of the rabies virus in the two locations of the brain and the glands as shown by the comparative effect of physical and chemical agents.

In conclusion, we wish to express our thanks to Dr. C. Zell, Frederick Jelinek, and Miss L. Alper, who assisted largely in the technical part of the work.